03-30-00 A

jc756 U.S. PTO 03/28/00

Practitioner's Docket No. 49674 **PATENT**

jc544 U.S. PTO 09/537858 03/28/00

Preliminary Classification:

Proposed Class:

Subclass:

NOTE: "All applicants are requested to include a preliminary classification on newly filed patent applications. The preliminary classification, preferably class and subclass designations, should be identified in the upper right-hand corner of the letter of transmittal accompanying the application papers, for example 'Proposed Class 2, subclass 129.'" M.P.E.P. § 601, 7th ed.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

NEW APPLICATION TRANSMITTAL

Transmitted herewith for filing is the patent application of

Inventor(s): Paul PROOST, Sofie STRUYF and Jo VAN DAMME

WARNING: *37 C.F.R. § 1.41(a)(1) points out:*

"(a) A patent is applied for in the name or names of the actual inventor or inventors.

"(1) The inventorship of a nonprovisional application is that inventorship set forth in the oath or declaration as prescribed by § 1.63, except as provided for in § 1.53(d)(4) and § 1.63(d). If an oath or declaration as prescribed by § 1.63 is not filed during the pendency of a nonprovisional application, the inventorship is that inventorship set forth in the application papers filed pursuant to § 1.53(b), unless a petition under this paragraph accompanied by the fee set forth in § 1.17(i) is filed supplying or changing the name or names of the inventor or inventors."

For (title): AMINO-TERMINALLY TRUNCATED RANTES AS CHEMOKINE ANTAGONISTS

CERTIFICATION UNDER 37 C.F.R. § 1.10*
(Express Mail label number is mandatory.)
(Express Mail certification is optional.)

I hereby certify that this New Application Transmittal and the documents referred to as attached therein are being deposited with the United States Postal Service on this date March 28, 2000 in an envelope as "Express Mail Post Office to Addressee," mailing Label Number TB553892394US, addressed to the: Assistant Commissioner for Patents, Washington, D.C. 20231.

Peter F. Corless
(type or print name of person mailing paper)

Signature of person mailing paper

WARNING: *Certificate of mailing (first class) or facsimile transmission procedures of 37 C.F.R. § 1.8 cannot be used to obtain a date of mailing or transmission for this correspondence.*

WARNING: *Each paper or fee filed by "Express Mail" must have the number of the "Express Mail" mailing label placed thereon prior to mailing. 37 C.F.R. § 1.10(b).*
"Since the filing of correspondence under § 1.10 without the Express Mail mailing label thereon is an oversight that can be avoided by the exercise of reasonable care, requests for waiver of this requirement will not be granted on petition." Notice of Oct. 24, 1996, 60 Fed. Reg. 56,439, at 56,442.

1. Type of Application

This new application is for a(n)

(check one applicable item below)

Original (nonprovisional)

☐ Design

☐ Plant

WARNING: ***Do not*** *use this transmittal for a completion in the U.S. of an International Application under 35 U.S.C. § 371(c)(4), unless the International Application is being filed as a divisional, continuation or continuation-in-part application.*

WARNING: ***Do not*** *use this transmittal for the filing of a provisional application.*

NOTE: If one of the following 3 items apply, then complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF A PRIOR U.S. APPLICATION CLAIMED and a NOTIFICATION IN PARENT APPLICATION OF THE FILING OF THIS CONTINUATION APPLICATION.

☐ Divisional.

☒ Continuation.

☐ Continuation-in-part (C-I-P).

2. Benefit of Prior U.S. Application(s) (35 U.S.C. §§ 119(e), 120, or 121)

NOTE: A nonprovisional application may claim an invention disclosed in one or more prior filed copending nonprovisional applications or copending international applications designating the United States of America. In order for a nonprovisional application to claim the benefit of a prior filed copending nonprovisional application or copending international application designating the United States of America, each prior application must name as an inventor at least one inventor named in the later filed nonprovisional application and disclose the named inventor's invention claimed in at least one claim of the later filed nonprovisional application in the manner provided by the first paragraph of 35 U.S.C. § 112. Each prior application must also be:

(i) An international application entitled to a filing date in accordance with PCT Article 11 and designating the United States of America; or

(ii) Complete as set forth in § 1.51(b); or

(iii) Entitled to a filing date as set forth in § 1.53(b) or § 1.53(d) and include the basic filing fee set forth in § 1.16; or

(iv) Entitled to a filing date as set forth in § 1.53(b) and have paid therein the processing and retention fee set forth in § 1.21(l) within the time period set forth in § 1.53(f).

37 C.F.R. § 1.78(a)(1).

NOTE: If the new application being transmitted is a divisional, continuation or a continuation-in-part of a parent case, or where the parent case is an International Application which designated the U.S., or benefit of a prior provisional application is claimed, then check the following item and complete and attach ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

WARNING: *When the last day of pendency of a provisional application falls on a Saturday, Sunday, or Federal holiday within the District of Columbia, any nonprovisional application claiming benefit of the provisional application **must** be filed prior to the Saturday, Sunday, or Federal holiday within the District of Columbia. See 37 C.F.R. § 1.78(a)(3).*

☒ The new application being transmitted claims the benefit of prior U.S. application(s). Enclosed are ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

3. Papers Enclosed

A. Required for filing date under 37 C.F.R. § 1.53(b) (Regular) or 37 C.F.R. § 1.153 (Design) Application

23 Pages of specification

2 Pages of claims

10 Sheets of drawing

WARNING: ***DO NOT*** *submit original drawings. A high quality copy of the drawings should be supplied when filing a patent application. The drawings that are submitted to the Office must be on strong, white, smooth, and non-shiny paper and meet the standards according to § 1.84. If corrections to the drawings are necessary, they should be made to the original drawing and a high-quality copy of the corrected original drawing then submitted to the Office. Only one copy is required or desired. For comments on proposed then-new 37 C.F.R. § 1.84, see Notice of March 9, 1988 (1990 O.G. 57-62).*

NOTE: "Identifying indicia, if provided, should include the application number or the title of the invention, inventor's name, docket number (if any), and the name and telephone number of a person to call if the Office is unable to match the drawings to the proper application. This information should be placed on the back of each sheet of drawing a minimum distance of 1.5 cm. (5/8 inch) down from the top of the page . . ." 37 C.F.R. § 1.84(c)).

(complete the following, if applicable)

☐ The enclosed drawing(s) are photograph(s), and there is also attached a "PETITION TO ACCEPT PHOTOGRAPH(S) AS DRAWING(S)." 37 C.F.R. § 1.84(b).

☐ formal

☐ informal

B. Other Papers Enclosed

____ Pages of declaration and power of attorney

1 Pages of abstract

2 Other Pages of Sequence Listing

4. Additional papers enclosed

☐ Amendment to claims

☐ Cancel in this applications claims ________________ before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

☐ Add the claims shown on the attached amendment. (Claims added have been numbered consecutively following the highest numbered original claims.)

☐ Preliminary Amendment

☐ Information Disclosure Statement (37 C.F.R. § 1.98)

☐ Form PTO-1449 (PTO/SB/08A and 08B)

☐ Citations

☐ Declaration of Biological Deposit

☐ Submission of "Sequence Listing," computer readable copy and/or amendment pertaining thereto for biotechnology invention containing nucleotide and/or amino acid sequence.

☐ Authorization of Attorney(s) to Accept and Follow Instructions from Representative

☐ Special Comments

Other

5. Declaration or oath (including power of attorney)

NOTE: A newly executed declaration is not required in a continuation or divisional application provided that the prior nonprovisional application contained a declaration as required, the application being filed is by all or fewer than all the inventors named in the prior application, there is no new matter in the application being filed, and a copy of the executed declaration filed in the prior application (showing the signature or an indication thereon that it was signed) is submitted. The copy must be accompanied by a statement requesting deletion of the names of person(s) who are not inventors of the application being filed. If the declaration in the prior application was filed under § 1.47, then a copy of that declaration must be filed accompanied by a copy of the decision granting § 1.47 status or, if a nonsigning person under § 1.47 has subsequently joined in a prior application, then a copy of the subsequently executed declaration must be filed. See 37 C.F.R. §§ 1.63(d)(1)–(3).

NOTE: A declaration filed to complete an application must be executed, identify the specification to which it is directed, identify each inventor by full name including family name and at least one given name, without abbreviation together with any other given name or initial, and the residence, post office address and country or citizenship of each inventor, and state whether the inventor is a sole or joint inventor. 37 C.F.R. § 1.63(a)(1)–(4).

☐ Enclosed

Executed by

(check **all** *applicable boxes)*

☐ inventor(s).

☐ legal representative of inventor(s). 37 C.F.R. §§ 1.42 or 1.43.

☐ joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached.

☐ This is the petition required by 37 C.F.R. § 1.47 and the statement required by 37 C.F.R. § 1.47 is also attached. See item 13 below for fee.

☒ Not Enclosed.

NOTE: Where the filing is a completion in the U.S. of an International Application or where the completion of the U.S. application contains subject matter in addition to the International Application, the application may be treated as a continuation or continuation-in-part, as the case may be, utilizing ADDED PAGE FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION CLAIMED.

☐ Application is made by a person authorized under 37 C.F.R. § 1.41(c) on behalf of *all* the above named inventor(s).

(The declaration or oath, along with the surcharge required by 37 C.F.R. § 1.16(e) can be filed subsequently).

☐ Showing that the filing is authorized. *(not required unless called into question. 37 C.F.R. § 1.41(d))*

6. Inventorship Statement

WARNING: *If the named inventors are each not the inventors of all the claims an explanation, including the ownership of the various claims at the time the last claimed invention was made, should be submitted.*

The inventorship for all the claims in this application are:

☐ The same.

or

☐ Not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made,

- ☐ is submitted.
- ☐ will be submitted.

7. Language

NOTE: *An application including a signed oath or declaration may be filed in a language other than English. An English translation of the non-English language application and the processing fee of $130.00 required by 37 C.F.R. § 1.17(k) is required to be filed with the application, or within such time as may be set by the Office. 37 C.F.R. § 1.52(d).*

☒ English

☐ Non-English

- ☐ The attached translation includes a statement that the translation is accurate. 37 C.F.R. § 1.52(d).

8. Assignment

☒ An assignment of the invention to Applied Research Systems of Curacao Netherlands Antilles

- ☐ is attached. A separate ☐ "COVER SHEET FOR ASSIGNMENT (DOCUMENT) ACCOMPANYING NEW PATENT APPLICATION" or ☐ FORM PTO 1595 is also attached.
- ☒ will follow.

NOTE: *"If an assignment is submitted with a new application, send two separate letters-one for the application and one for the assignment." Notice of May 4, 1990 (1114 O.G. 77-78).*

WARNING: *A newly executed "CERTIFICATE UNDER 37 C.F.R. § 3.73(b)" must be filed when a continuation-in-part application is filed by an assignee. Notice of April 30, 1993, 1150 O.G. 62-64.*

9. Certified Copy

Certified copy(ies) of application(s)

Country	Appln. No.	Filed
Europe	97116863.8	29 September 1997
Europe	97122471.2	19 December 1997
Europe	98104216.1	10 March 1998

from which priority is claimed

☐ is (are) attached.

☒ will follow.

NOTE: The foreign application forming the basis for the claim for priority must be referred to in the oath or declaration. 37 C.F.R. § 1.55(a) and 1.63.

NOTE: This item is for any foreign priority for which the application being filed directly relates. If any parent U.S. application or International Application from which this application claims benefit under 35 U.S.C. § 120 is itself entitled to priority from a prior foreign application, then complete item 18 on the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED.

10. Fee Calculation (37 C.F.R. § 1.16)

A. ☒ Regular application

CLAIMS AS FILED				
Number filed	Number Extra		Rate	Basic Fee 37 C.F.R. § 1.16(a) $760.00
Total Claims (37 C.F.R. § 1.16(c))	18 − 20 =	×	$ 18.00	
Independent Claims (37 C.F.R. § 1.16(b))	2 − 3 =	×	$ 78.00	
Multiple dependent claim(s), if any (37 C.F.R. § 1.16(d))		+	$260.00	260.00

☐ Amendment cancelling extra claims is enclosed.

☐ Amendment deleting multiple-dependencies is enclosed.

☐ Fee for extra claims is not being paid at this time.

NOTE: If the fees for extra claims are not paid on filing they must be paid or the claims cancelled by amendment, prior to the expiration of the time period set for response by the Patent and Trademark Office in any notice of fee deficiency. 37 C.F.R. § 1.16(d).

Filing Fee Calculation $ 950.00

B. ☐ Design application ($310.00—37 C.F.R. § 1.16(f))

Filing Fee Calculation $_____

C. ☐ Plant application ($480.00—37 C.F.R. § 1.16(g))

Filing fee calculation $_____

11. Small Entity Statement(s)

☐ Statement(s) that this is a filing by a small entity under 37 C.F.R. § 1.9 and 1.27 is (are) attached.

WARNING: *"Status as a small entity must be specifically established in each application or patent in which the status is available and desired. Status as a small entity in one application or patent does not affect any other application or patent, including applications or patents which are directly or indirectly dependent upon the application or patent in which the status has been established. The refiling of an application under § 1.53 as a continuation, division, or continuation-in-part (including a continued prosecution application under § 1.53(d)), or the filing of a reissue application requires a new determination as to continued entitlement to small entity status for the continuing or reissue application. A nonprovisional application claiming benefit under 35 U.S.C. § 119(e), 120, 121, or 365(c) of a prior application, or a reissue application may rely on a statement filed in the prior application or in the patent if the nonprovisional application or the reissue application includes a reference to the statement in the prior application or in the patent or includes a copy of the statement in the prior application or in the patent and status as a small entity is still proper and desired. The payment of the small entity basic statutory filing fee will be treated as such a reference for purposes of this section." 37 C.F.R. § 1.28(a)(2).*

WARNING: *"Small entity status must not be established when the person or persons signing the . . . statement can* ***unequivocally*** *make the required self-certification." M.P.E.P., § 509.03, 6th ed., rev. 2, July 1996 (emphasis added).*

(complete the following, if applicable)

☐ Status as a small entity was claimed in prior application

_____ / _____, filed on _____, from which benefit is being claimed for this application under:

35 U.S.C. § ☐ 119(e),
☐ 120,
☐ 121,
☐ 365(c),

and which status as a small entity is still proper and desired.

☐ A copy of the statement in the prior application is included.

Filing Fee Calculation (50% of **A, B** or **C** above)

$_____

NOTE: Any excess of the full fee paid will be refunded if small entity status is established and a refund request are filed within 2 months of the date of timely payment of a full fee. The two-month period is not extendable under § 1.136. 37 C.F.R. § 1.28(a).

12. Request for International-Type Search (37 C.F.R. § 1.104(d))

(complete, if applicable)

☐ Please prepare an international-type search report for this application at the time when national examination on the merits takes place.

13. Fee Payment Being Made at This Time

☒ Not Enclosed

☒ No filing fee is to be paid at this time.
(This and the surcharge required by 37 C.F.R. § 1.16(e) can be paid subsequently.)

☐ Enclosed

☐ Filing fee $ _____

☐ Recording assignment
($40.00; 37 C.F.R. § 1.21(h))
(See attached "COVER SHEET FOR ASSIGNMENT ACCOMPANYING NEW APPLICATION".) $ _____

☐ Petition fee for filing by other than all the inventors or person on behalf of the inventor where inventor refused to sign or cannot be reached
($130.00; 37 C.F.R. §§ 1.47 and 1.17(i)) $ _____

☐ For processing an application with a specification in a non-English language
($130.00; 37 C.F.R. §§ 1.52(d) and 1.17(k)) $ _____

☐ Processing and retention fee
($130.00; 37 C.F.R. §§ 1.53(d) and 1.21(l)) $ _____

☐ Fee for international-type search report
($40.00; 37 C.F.R. § 1.21(e)) $ _____

NOTE: 37 C.F.R. § 1.21(l) establishes a fee for processing and retaining any application that is abandoned for failing to complete the application pursuant to 37 C.F.R. § 1.53(f) and this, as well as the changes to 37 C.F.R. §§ 1.53 and 1.78(a)(1), indicate that in order to obtain the benefit of a prior U.S. application, either the basic filing fee must be paid, or the processing and retention fee of § 1.21(l) must be paid, within 1 year from notification under § 53(f).

Total fees enclosed $_____

14. Method of Payment of Fees

☐ Check in the amount of $_____

☐ Charge Account No. _____ in the amount of $_____.
A duplicate of this transmittal is attached.

NOTE: Fees should be itemized in such a manner that it is clear for which purpose the fees are paid. 37 C.F.R. § 1.22(b).

15. Authorization to Charge Additional Fees

WARNING: *If no fees are to be paid on filing, the following items should not be completed.*

WARNING: *Accurately count claims, especially multiple dependent claims, to avoid unexpected high charges, if extra claim charges are authorized.*

☐ The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Account No. _____:

☐ 37 C.F.R. § 1.16(a), (f) or (g) (filing fees)

☐ 37 C.F.R. § 1.16(b), (c) and (d) (presentation of extra claims)

NOTE: Because additional fees for excess or multiple dependent claims not paid on filing or on later presentation must only be paid or these claims cancelled by amendment prior to the expiration of the time period set for response by the PTO in any notice of fee deficiency (37 C.F.R. § 1.16(d)), it might be best not to authorize the PTO to charge additional claim fees, except possibly when dealing with amendments after final action.

☐ 37 C.F.R. § 1.16(e) (surcharge for filing the basic filing fee and/or declaration on a date later than the filing date of the application)

☐ 37 C.F.R. § 1.17(a)(1)–(5) (extension fees pursuant to § 1.136(a)).

☐ 37 C.F.R. § 1.17 (application processing fees)

NOTE: ". . .A written request may be submitted in an application that is an authorization to treat any concurrent or future reply, requiring a petition for an extension of time under this paragraph for its timely submission, as incorporating a petition for extension of time for the appropriate length of time. An authorization to charge all required fees, fees under § 1.17, or all required extension of time fees will be treated as a constructive petition for an extension of time in any concurrent or future reply requiring a petition for an extension of time under this paragraph for its timely submission. Submission of the fee set forth in § 1.17(a) will also be treated as a constructive petition for an extension of time in any concurrent reply requiring a petition for an extension of time under this paragraph for its timely submission." 37 C.F.R. § 1.136(a)(3).

☐ 37 C.F.R. § 1.18 (issue fee at or before mailing of Notice of Allowance, pursuant to 37 C.F.R. § 1.311(b))

NOTE: Where an authorization to charge the issue fee to a deposit account has been filed before the mailing of a Notice of Allowance, the issue fee will be automatically charged to the deposit account at the time of mailing the notice of allowance. 37 C.F.R. § 1.311(b).

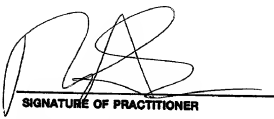
NOTE: 37 C.F.R. § 1.28(b) requires "Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application . . . prior to paying, or at the time of paying, . . . the issue fee. . . " From the wording of 37 C.F.R. § 1.28(b), (a) notification of change of status must be made even if the fee is paid as "other than a small entity" and (b) no notification is required if the change is to another small entity.

16. Instructions as to Overpayment

NOTE: ". . . Amounts of twenty-five dollars or less will not be returned unless specifically requested within a reasonable time, nor will the payer be notified of such amounts; amounts over twenty-five dollars may be returned by check or, if requested, by credit to a deposit account." 37 C.F.R. § 1.26(a).

☐ Credit Account No. _____

☐ Refund



SIGNATURE OF PRACTITIONER

Reg. No. 33,860

Peter F. Corless

(type or print name of attorney)

Tel. No. (617) 523-3400

Dike, Bronstein, Roberts & Cushman, LLP

130 Water Street

P.O. Address

Customer No.

Boston, MA 02109

☒ **Incorporation by reference of added pages**

(check the following item if the application in this transmittal claims the benefit of prior U.S. application(s) (including an international application entering the U.S. stage as a continuation, divisional or C-I-P application) and complete and attach the ADDED PAGES FOR NEW APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED)

☒ Plus Added Pages for New Application Transmittal Where Benefit of Prior U.S. Application(s) Claimed

Number of pages added 5

☐ Plus Added Pages for Papers Referred to in Item 4 Above

Number of pages added _____

☐ Plus added pages deleting names of inventor(s) named in prior application(s) who is/are no longer inventor(s) of the subject matter claimed in this application.

Number of pages added _____

☐ Plus "Assignment Cover Letter Accompanying New Application"

Number of pages added _____

☐ **Statement Where No Further Pages Added**

(if no further pages form a part of this Transmittal, then end this Transmittal with this page and check the following item)

☐ This transmittal ends with this page.

Practitioner's Docket No. 49674 **PATENT**

ADDED PAGES FOR APPLICATION TRANSMITTAL WHERE BENEFIT OF PRIOR U.S. APPLICATION(S) CLAIMED

NOTE: See 37 C.F.R. § 1.78.

17. Relate Back

WARNING: *If an application claims the benefit of the filing date of an earlier filed application under 35 U.S.C. §§ 120, 121 or 365(c), the 20-year term of that application will be based upon the filing date of the earliest U.S. application that the application makes reference to under 35 U.S.C. §§ 120, 121 or 365(c). (35 U.S.C. § 154(a)(2) does not take into account, for the determination of the patent term, any application on which priority is claimed under 35 U.S.C. §§ 119, 365(a) or 365(b).) For a c-i-p application, applicant should review whether any claim in the patent that will issue is supported by an earlier application and, if not, the applicant should consider canceling the reference to the earlier filed application. The term of a patent is not based on a claim-by-claim approach. See Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,205.*

(complete the following, if applicable)

☒ Amend the specification by inserting, before the first line, the following sentence:

A. 35 U.S.C. § 119(e)

NOTE: "Any nonprovisional application claiming the benefit of one or more prior filed copending provisional applications must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior provisional application, identifying it as a provisional application, and including the provisional application number (consisting of series code and serial number)." 37 C.F.R. § 1.78(a)(4).

☐ "This application claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	FILING DATE
_____ /_____	_____ "
_____ /_____	_____ "
_____ /_____	_____ "

B. 35 U.S.C. §§ 120, 121 and 365(c)

NOTE: *"Except for a continued prosecution application filed under § 1.53(d), any nonprovisional application claiming the benefit of one or more prior filed copending nonprovisional applications or international applications designating the United States of America must contain or be amended to contain in the first sentence of the specification following the title a reference to each such prior application, identifying it by application number (consisting of the series code and serial number) or international application number and international filing date and indicating the relationship of the applications. . . . Cross-references to other related applications may be made when appropriate." (See § 1.14(a)). 37 C.F.R. § 1.78(a)(2).*

☒ "This application is a

☒ continuation

☐ continuation-in-part

☐ divisional

of copending application(s)

☐ application number 0 /__________ filed on __________"

☒ International Application PCT/EP98/06143 filed on 28 September 1998 and which designated the U.S."

NOTE: *The proper reference to a prior filed PCT application that entered the U.S. national phase is the U.S. serial number and the filing date of the PCT application that designated the U.S.*

NOTE: *(1) Where the application being transmitted adds subject matter to the International Application, then the filing can be as a continuation-in-part or (2) if it is desired to do so for other reasons then the filing can be as a continuation.*

NOTE: *The deadline for entering the national phase in the U.S. for an international application was clarified in the Notice of April 28, 1987 (1079 O.G. 32 to 46) as follows:*

"The Patent and Trademark Office considers the International application to be pending until the 22nd month from the priority date if the United States has been designated and no Demand for International Preliminary Examination has been filed prior to the expiration of the 19th month from the priority date and until the 32nd month from the priority date if a Demand for International Preliminary Examination which elected the United States of America has been filed prior to the expiration of the 19th month from the priority date, provided that a copy of the international application has been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively. If a copy of the international application has not been communicated to the Patent and Trademark Office within the 20 or 30 month period respectively, the international application becomes abandoned as to the United States 20 or 30 months from the priority date respectivley. These periods have been placed in the rules as paragraph (h) of § 1.494 and paragraph (i) of § 1.495. A continuing application under 35 U.S.C. 365(c) and 120 may be filed anytime during the pendency of the international application."

☐ "The nonprovisional application designated above, namely application ____ / __________, filed ______, claims the benefit of U.S. Provisional Application(s) No(s).:

APPLICATION NO(S).:	FILING DATE
____ / __________	__________"
____ / __________	__________"
____ / __________	__________"

☐ Where more than one reference is made above, please combine all references into one sentence.

18. Relate Back—35 U.S.C. § 119 Priority Claim for Prior Application

The prior U.S. application(s), including any prior International Application designating the U.S., identified above in Item 17B, in turn itself claim(s) foreign priority(ies) as follows:

Country	Appln. no.	Filed on
International	PCT/EP98/06143	September 28, 1998

The certified copy(ies) has (have)

☐ been filed on _____, in prior application 0 /_____, which was filed on _____.

☐ is (are) attached.

WARNING: *The certified copy of the priority application that may have been communicated to the PTO by the International Bureau may **not** be relied on without any need to file a certified copy of the priority application **in the continuing application.** This is so because the certified copy of the priority application communicated by the International Bureau is placed in a folder and is not assigned a U.S. serial number unless the national stage is entered. Such folders are disposed of if the national stage is not entered. Therefore, such certified copies may not be available if needed later in the prosecution of a continuing application. An alternative would be to physically remove the priority documents from the folders and transfer them to the continuing application. The resources required to request transfer, retrieve the folders, make suitable record notations, transfer the certified copies, enter and make a record of such copies in the Continuing Application are substantial. Accordingly, the priority documents in folders of international applications that have not entered the national stage may not be relied on. Notice of April 28, 1987 (1079 O.G. 32 to 46).*

19. Maintenance of Copendency of Prior Application

NOTE: The PTO finds it useful if a copy of the petition filed in the prior application extending the term for response is filed with the papers constituting the filing of the continuation application. Notice of November 5, 1985 (1060 O.G. 27).

A. ☐ Extension of time in prior application

*(This item **must** be completed and the papers filed **in the prior application,** if the period set in the prior application has run.)*

☐ A petition, fee and response extends the term in the pending **prior** application until _____

☐ A **copy** of the petition filed in prior application is attached.

B. ☐ Conditional Petition for Extension of Time in Prior Application

(complete this item, if previous item not applicable)

☐ A conditional petition for extension of time is being filed in the **pending prior** application.

☐ A **copy** of the conditional petition filed in the prior application is attached.

20. Further Inventorship Statement Where Benefit of Prior Application(s) Claimed

(complete applicable item (a), (b) and/or (c) below)

(a) ☐ This application discloses and claims only subject matter disclosed in the prior application whose particulars are set out above and the inventor(s) in this application are

☐ the same.

☐ less than those named in the prior application. It is requested that the following inventor(s) identified for the prior application be deleted:

____
(type name(s) of inventor(s) to be deleted)

(b) ☐ This application discloses and claims additional disclosure by amendment and a new declaration or oath is being filed. With respect to the prior application, the inventor(s) in this application are

☐ the same.

☐ the following additional inventor(s) have been added:

____
(type name(s) of inventor(s) to be added)

(c) The inventorship for all the claims in this application are

☐ the same.

☐ not the same. An explanation, including the ownership of the various claims at the time the last claimed invention was made

☐ is submitted.

☐ will be submitted.

21. Abandonment of Prior Application *(if applicable)*

☐ Please abandon the prior application at a time while the prior application is pending, or when the petition for extension of time or to revive in that application is granted, and when this application is granted a filing date, so as to make this application copending with said prior application.

NOTE: *According to the Notice of May 13, 1983 (103, TMOG 6-7), the filing of a continuation or continuation-in-part application is a proper response with respect to a petition for extension of time or a petition to revive and should include the express abandonment of the prior application conditioned upon the granting of the petition and the granting of a filing date to the continuing application.*

22. Petition for Suspension of Prosecution for the Time Necessary to File an Amendment

WARNING: *"The claims of a new application may be finally rejected in the first Office action in those situations where (A) the new application is a continuing application of, or a substitute for, an earlier application, and (B) all the claims of the new application (1) are drawn to the same invention claimed in the earlier application, and (2) would have been properly finally rejected on the grounds of art of record in the next Office action if they had been entered in the earlier application." M.P.E.P., § 706.07(b), 7th ed.*

NOTE: *Where it is possible that the claims on file will give rise to a first action final for this continuation application and for some reason an amendment cannot be filed promptly (e.g., experimental data is being gathered) it may be desirable to file a petition for suspension of prosecution for the time necessary.*

(check the next item, if applicable)

☐ There is provided herewith a Petition To Suspend Prosecution for the Time Necessary to File An Amendment (New Application Filed Concurrently)

23. Small Entity (37 C.F.R. § 1.28(a))

☐ Applicant has established small entity status by the filing of a statement in parent application /__________ on __________ .

☐ A copy of the statement previously filed is included.

WARNING: *See 37 C.F.R. § 1.28(a).*

WARNING: *"Small entity status must not be established when the person or persons signing the . . . statement can* ***unequivocally*** *make the required self-certification." M.P.E.P., § 509.03, 7th ed. (emphasis added).*

24. NOTIFICATION IN PARENT APPLICATION OF THIS FILING

☐ A notification of the filing of this *(check one of the following)*

☐ continuation

☐ continuation-in-part

☐ divisional

is being filed in the parent application, from which this application claims priority under 35 U.S.C. § 120.

Docket No. 49674
Express Mail Label No. TB553892394US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
NEW PATENT APPLICATION

TITLE: AMINO-TERMINALLY TRUNCATED RANTES AS CHEMOKINE ANTAGONISTS

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AMINO-TERMINALLY TRUNCATED RANTES AS CHEMOKINE ANTAGONISTS

FIELD OF THE INVENTION

The present invention relates to amino-terminally truncated RANTES, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3 or 1-4 of the naturally-occurring RANTES and having chemokine antagonistic activity, as well as cDNA sequences encoding them, their use in therapy and/or in diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required, and pharmaceutical compositions comprising them.

BACKGROUND OF THE INVENTION

Chemokines constitute a family of small pro-inflammatory cytokines with leukocyte chemotactic and activating properties. Depending on the position of the first cysteines, the chemokine family can be divided in C-C, C-X-C and C-X_3-C chemokines (Baggiolini M. et al., 1994; Baggiolini M. et al., 1997 and Taub D. et al., 1996).

Many C-X-C chemokines such as interleukin-8 (IL-8) are chemotactic for neutrophils, while C-C chemokines, such as monocyte chemotactic protein-3 (MCP-3), are active on a variety of leukocytes including monocytes, lymphocytes, eosinophils, basophils, NK cells and dendritic cells.

The NH_2 terminal domain of chemokines is involved in receptor-binding and NH_2-terminal processing can activate chemokines, reduce their chemokine activity or render chemokines completely inactive.

The C-X-C chemokine platelet basic protein becomes a neutrophil chemotactic peptide (NAP-2) only after removal of the 24 NH_2-terminal residues (Walz A. et al., 1989 and Van Damme J. et al., 1990).

Deletion of up to 8 NH_2-terminal residues from IL-8 results in an enhanced chemotactic activity, but further cleavage of the Glu-Leu-Arg motif, which is located in front of the first Cys in all neutrophil chemotactic C-X-C chemokines, causes complete inactivation (Clark-Lewis I. et al., 1991).

Similar NH_2-terminal proteolysis (up to 8 amino acids) of another C-X-C chemokine, granulocyte chemotactic protein-2 (GCP-2), has no effect on the neutrophil chemotactic activity (Proost P.et al, 1993a).

RANTES (is an acronym for "Regulated upon Activation, Normally T Expressed, and presumably Secreted") is a C-C chemokine, whose cDNA clone has been isolated from a cDNA library enriched for T cell-specific sequences (Schall T. J. et al., 1988).

The synthetical C-C chemokines MCP-1, MCP-3 and RANTES missing the 8 to 9 NH_2-terminal amino acids are inactive on monocytes and are useful as receptor antagonists (Gong.J. et al., 1996; and Gong J. et al., 1995).

Extension of RANTES with one methionine results in complete inactivation of the molecule and Met-RANTES behaves as an antagonist for the authentic RANTES (Proudfoot A.E. et al., 1996).

DESCRIPTION OF THE INVENTION

The main object of the present invention is amino-terminally truncated RANTES, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3 or 1-4 of the naturally-occurring RANTES and having chemokine antagonistic activity.

A particular object of the present invention is RANTES(3-68), which is RANTES lacking the first 2 amino acids, as shown in Figure 1 and in SEQ ID NO: 2.

The amino-terminally truncated RANTES of the invention can be in a glycosylated or non-glycosylated form.

The term "chemokine antagonist" means 'which acts as antagonist to the mature full-length naturally-occurring chemokines'.

Another object of the invention are the DNA molecules comprising the DNA sequences coding for the amino-terminally truncated RANTES of the invention, including nucleotide sequences substantially the same. The cDNA sequence of intact RANTES is disclosed in Schall T. J. et al. (1988) and the cDNA of the truncated RANTES can be easily deduced.

"Nucleotide sequences substantially the same" includes all other nucleic acid sequences which, by virtue of the degeneracy of the genetic code, also code for the given amino acid sequences.

The invention also includes expression vectors which comprise the above DNAs, host-cells transformed with such vectors and a process of preparation of such amino-terminally truncated RANTES of the invention, through the culture in appropriate culture media of said transformed cells.

The DNA sequence coding for the proteins of the invention can be inserted and ligated into a suitable plasmid. Once formed, the expression vector is introduced into a suitable host cell, which then expresses the vector(s) to yield the desired protein.

Expression of any of the recombinant proteins of the invention as mentioned herein can be effected in eukaryotic cells (e.g. yeasts, insect or mammalian cells) or prokaryotic cells, using the appropriate expression vectors. Any method known in the art can be employed.

For example the DNA molecules coding for the proteins obtained by any of the above methods are inserted into appropriately constructed expression vectors by techniques well known in the art (see Sambrook et al, 1989). Double stranded cDNA is linked to plasmid vectors by homopolymeric tailing or by restriction linking involving the use of synthetic DNA linkers or blunt-ended ligation techniques: DNA ligases are used to ligate the DNA molecules and undesirable joining is avoided by treatment with alkaline phosphatase.

In order to be capable of expressing the desired protein, an expression vector should also comprise specific nucleotide sequences containing transcriptional and translational regulatory information linked to the DNA coding the desired protein in such a way as to permit gene expression and production of the protein. First in order for the gene to be transcribed, it must be preceded by a promoter recognizable by RNA polymerase, to which the polymerase binds and thus initiates the transcription process. There are a variety of such promoters in use, which work with different efficiencies (strong and weak promoters).

For eukaryotic hosts, different transcriptional and translational regulatory sequences may be employed, depending on the nature of the host. They may be derived form viral sources, such as adenovirus, bovine papilloma virus, Simian virus or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Examples are the TK promoter of the Herpes virus, the SV40 early

promoter, the yeast gal4 gene promoter, etc. Transcriptional initiation regulatory signals may be selected which allow for repression and activation, so that expression of the genes can be modulated.

The DNA molecule comprising the nucleotide sequence coding for the protein of the invention is inserted into vector(s), having the operably linked transcriptional and translational regulatory signals, which is capable of integrating the desired gene sequences into the host cell.

The cells which have been stably transformed by the introduced DNA can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may also provide for phototrophy to a auxotropic host, biocide resistance, e.g. antibiotics, or heavy metals such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of proteins of the invention.

Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells, that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Once the vector(s) or DNA sequence containing the construct(s) has been prepared for expression the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means: transformation, transfection, conjugation, protoplast fusion, electroporation, calcium phosphate-precipitation, direct microinjection, etc.

Host cells may be either prokaryotic or eukaryotic. Preferred are eukaryotic hosts, e.g. mammalian cells, such as human, monkey, mouse, and Chinese hamster ovary (CHO) cells, because they provide post-translational modifications to protein molecules, including correct folding or glycosylation at correct sites. Also yeast cells can carry out post-translational peptide modifications including glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high

copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

After the introduction of the vector(s), the host cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the desired proteins.

The amino-terminally truncated RANTES of the invention may be prepared by any other well known procedure in the art, in particular, by the well established chemical synthesis procedures, utilizing automated solid-phase peptide synthesizers followed by chromatographic purification.

The chemokines of the invention may, for example, be synthesized by Fmoc (9-fluorenylmethoxycarbonyl), tBoc (t-butoxycarbonyl) or any other comparable chemical synthesis with or without appropriate side-chain protection groups on the different amino acids. The amino acids with or without appropriate side-chain protection groups are preactivated - e.g. with HBTU/HOBt [2-(1H-Benzotriazole-1yl)-1,1,3,3-tetramethyl-uromium hexafluorophosphate/1-hydroxybenzotriazole) - and coupled to the growing peptide chain. Before the addition of the following residue, the protection group (e.g. Fmoc) is removed from the α-amino group. After synthesis, all protection groups are removed, the intact full length peptides are purified and chemically or enzymatically folded (including the formation of disulphide bridges between cysteines) into the corresponding chemokines of the invention.

Purification of the natural, synthetic or recombinant proteins is carried out by any one of the methods known for this purpose, i.e. any conventional procedure involving extraction, precipitation, chromatography, electrophoresis, or the like (see for example Proost P. et al., 1996). A further purification procedure that may be used in preference for purifying the protein of the invention is affinity chromatography using monoclonal antibodies, or affinity for heparin, which bind the target protein and which are produced and immobilized on a gel matrix contained within a column. Impure preparations containing the proteins are passed through the column. The protein will be bound to the column by the specific antibody while the impurities will pass through. After washing, the protein is eluted from the gel by a change in pH or ionic strength.

The amino-terminally truncated RANTES of the invention are useful in the therapy and/or diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required. Examples of such diseases include: inflammatory diseases, angiogenesis- and hematopoiesis-related diseases, tumors, infectious diseases, including HIV, auto-immune diseases, atherosclerosis, pulmonary diseases and skin disorders. The preferred use is in the field of HIV-infection.

Therefore, in a further aspect, the present invention provides the use of the protein of the invention in the manufacture of a medicament for the treatment of the above-mentioned diseases.

The medicament is preferably presented in the form of a pharmaceutical composition comprising the proteins of the invention together with one or more pharmaceutically acceptable carriers and/or excipients. Such pharmaceutical compositions form yet a further aspect of the present invention.

A further embodiment of the invention is the method of treatment of the above-mentioned diseases comprising administering a pharmacologically active amount of the amino-terminally truncated RANTES of the invention to subjects at risk of developing such diseases or to subjects already showing such pathologies.

It has also been found that CD26/DPP IV is able to generate NH_2-terminally truncated RANTES in vitro. RANTES is the first cytokine reported whose biological activity can be modified by CD26/DPP IV.

Therefore, another object of the present invention is the use of CD26/DPP IV in the therapy and/or diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required, with particular focus on inflammatory, immune and infectious diseases.

Since this represents the first example of an identified mechanism for endogeneously regulated chemokine modification into an antagonist, similar physiological processing, but mediated by other factors (proteases). The use of such factors (proteases) in the therapy and/or diagnosis of the above diseases is also included in this invention.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

DESCRIPTION OF THE FIGURES

Figure 1: it shows the amino acid sequences of RANTES. Signal sequences are reported in *italics*, whereas C -residues are in **bold**. Arrows indicate the first amino acids of the amino-terminally truncated RANTES of the invention, also called RANTES(3-68).

Figure 2: The chemotactic potencies of intact and NH_2-terminally truncated forms of natural or recombinant RANTES for monocytic THP-1 cells were compared in the Boyden microchamber assay: Natural RANTES(1-68) (Δ), natural, truncated RANTES(3-68) (□), intact recombinant RANTES(1-68) (▲) and CD26/DPP IV cleaved recombinant RANTES(3-68) (■). Results represent the mean chemotactic index ± SEM of four or more independent experiments.

Figure 3: Effect of natural RANTES(3-68) (□), natural RANTES(1-68) (Δ), recombinant RANTES(1-68) (▲) and recombinant CD26/DPP IV treated RANTES(3-68) (■) on the $[Ca^{2+}]_i$ in THP-1 cells. Results represent the mean increase in $[Ca^{2+}]_i$ ± SEM of three or more independent experiments.

Figure 4: It shows the desensitization of the Ca^{2+}-mobilizing activity of intact recRANTES(1-68) by RANTES(3-68). THP-1 cells were first stimulated with buffer or different concentrations of recombinant RANTES(1-68) or RANTES(3-68). Results represent the mean ± SEM (three or more independent experiments) increase in $[Ca^{2+}]_i$ in response to 30 ng/ml of intact recombinant RANTES as a second stimulus.

Figure 5: Comparison of the chemotactic potency of truncated RANTES(3-68) with intact RANTES(1-68). The eosinophilic granulocyte chemotactic activity of natural (nat) and recombinant (rec) truncated RANTES, intact RANTES and synthetic MCP-3 was determined in the microchamber assay. Results represent the mean chemotactic index (CI) ± SEM of two or more independent experiments (each performed in triplicate).

Figure 6: Desensitization of calcium mobilization by intact RANTES in CCR transfectants. Calcium mobilization experiments were performed in HOS cells transfected with CD4 and the CC chemokine receptors CCR1 or CCR5. Cells were first stimulated

with different concentrations of intact or truncated RANTES, followed by stimulation with 100 ng/ml of intact RANTES. The percentage inhibition of the $[Ca^{2+}]_i$ increase induced by the second stimulus is shown. This percentage was calculated by comparing the response of 100 ng/ml of intact RANTES after addition of RANTES(1-68) or RANTES(3-68) with the reponse after stimulation with buffer (100%). Results represent the mean percentage inhibition ± SEM of two or more experiments.

Figure 7: Potent inhibitory effect of RANTES (3-68) on infection of mononuclear cells by HIV-1. PHA-activated PBMC were infected with M-tropic HIV-1 Ba-L strain in the presence of various concentrations of RANTES (1-68) or RANTES (3-68) (0 to 1,000 ng/ml added at the time of infection). After ten days virus yields were monitored in the cell supernatant by a p-24 Ag ELISA (one representative experiment out of four is shown).

Figure 8: Effects of RANTES(1-68) and RANTES(3-68) on infection by the HIV-1 SF162 strain in PHA-activated PBMC. Virus yields were monitored 10 days after infection by a p24 Ag ELISA on the cell supernatant. Results of a representative experiment out of three are shown. * Under the detection limit of the p24 Ag ELISA (<5 pg/ml).

Figure 9: Expression of CD26 on HOS.CD4.CCR5 cells, U87.CD4.CCR5 cells and freshly-isolated PBMC. The percentage (%) of CD26 positive cells is indicated in each histogram.

Figure 10: Effects of RANTES(1-68), RANTES(1-68) plus sCD26 (50 U/L), and RANTES(3-68) on infection of HOS.CD4.CCR5 cells by the HIV-1 BaL strain. Virus yields were monitored in the cell supernatant 8 days after infection by a p24Ag ELISA. Results of a representative experiment out of three is shown.

EXAMPLES

EXAMPLE 1: Amino-terminally truncated RANTES

Material and Methods

Reagents

Natural human RANTES was produced by human Malavu hepatosarcoma cells, MG-63 osteosarcoma cells or peripheral blood leukocytes (Blood transfusion centers of

Antwerp and Leuven) and purified as previously described (Proost P. et al., 1996 and Proost P. et al., 1993). MCP-2, MCP-3 and GCP-2 were synthesized by Fmoc chemistry (Proost P. et al., 1995 and Wuyts A. et al., 1997), recombinant human RANTES was obtained from Peprotech (Rocky Hill, NJ) and recombinant MCP-1 was a gift from Dr. J.J. Oppenheim (NCI-NIH, Frederick, MD).

Human osteosarcoma (HOS) cells transfected with CD4 and one of the CC chemokine receptors CCR1, CCR3 or CCR5 (Deng H., et al., 1996) were grown in DMEM with glutamax. Puromycin (1 µg/ml) was added to the medium as a selection agent. All growth media (Gibco BRL/Life Technologies, Paisley, UK) were enriched with 10% FCS.

Human CD26/DPP IV was obtained from prostasomes, prostate derived organelles, which occur freely in seminal plasma. The enzyme was purified to homogeneity as described before using ion exchange followed by affinity chromatography onto adenosine deaminase (De Meester I. et al., 1996).

Incubation of chemokines with CD26/DPP IV and detection of proteolytic processing

A 100 to 1000 molar excess of chemokine was incubated overnight with CD26/DPP IV in 100 mM Tris/HCl pH 7.7. Chemokines were separated from CD26/DPP IV by SDS-PAGE on a Tris/Tricine gel system as previously described (Proost P. et al., 1996).

Proteins were electroblotted on PVDF (polyvinylidene fluoride) membranes (Problott, Perkin Elmer, Foster City, CA) and stained with coomassie brilliant blue R250. After destaining, membranes were rinsed at least 5 times with ultrapure water (Milli Q; Millipore, Bedford, MA).

To obtain sufficient amounts of pure truncated chemokine for biological assays, about 50 µg of recombinant chemokine was treated with CD26/DPP IV and the cleavage product was acidified with 0.1 % trifluoroacetic acid (TFA). Tween 20 (0.01 %) was added to prevent the chemokines from sticking to the tubes.

Chemokines were separated from CD26/DPP IV in an acetonitrile gradient on a C-8 Aquapore RP-300 column (1 x 50 mm) (Perkin Elmer). Fractions containing proteins were analyzed by SDS-PAGE and silver stained as described.

CD26/DPP IV treated chemokines, purified by RP-HPLC or excised from PVDF blots, were NH_2-terminally sequenced by Edman degradation on a pulsed liquid phase 477A/120A protein sequencer (Perkin Elmer) using *N*-methylpiperidine as a coupling base.

Detection of chemotactic activity

Chemokines were tested for their chemotactic potency on freshly isolated peripheral blood neutrophilic granulocytes (10^6 cells/ml) or cultured monocytic THP-1 cells (0.5 x 10^6 cells/ml) in the Boyden microchamber (Proost P. et al., 1996 and Proost P. et al., 1993).

After 45 min (granulocytes) or 2 h (THP-1 cells) incubation at 37 °C, the cells were fixed and stained. The cells that migrated through the 5 µm pore size polycarbonate membranes were counted microscopically in ten oil immersion fields.

The chemotactic index (C.I.) of a sample (triplicates in each chamber) was calculated as the number of cells that migrated to the test sample divided by the number of cells that migrated to control medium. In desensitization experiments, cells were incubated with biologically inactive chemokine-variants for 10 min at 37 °C before transfer to the chamber.

The percentage inhibition of the C.I. obtained by desensitization with HBSS-treated control cells was calculated for the evaluation of chemotaxis desensitization.

Detection of intracellular Ca^{2+} concentrations

Intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) were measured as previously described (Wuyts A., et al., 1997). Briefly, purified cells were incubated with the fluorescent indicator fura-2 (2.5 µM fura-2/AM, Molecular Probes Europe BV, Leiden, The Netherlands) and 0.01 % Pluronic F-127 (Sigma, St. Louis, MO).

After 30 min, cells were washed twice, resuspended in HBSS with 1 mM Ca^{2+} and incubated for 10 min at 37 °C before fura-2 fluorescence was measured in an LS50B

luminescence spectrophotometer (Perkin Elmer). Upon excitation at 340 and 380 nm, fluorescence was detected at 510 nm. The $[Ca^{2+}]_i$ was calculated from the Grynkiewicz equation (Grynkiewicz G. et al., 1985).

In order to determine R_{max}, the cells were lysed with 50 µM digitonin. Subsequently, the pH was adjusted to 8.5 with 20 mM Tris and R_{min} was obtained by addition of 10 mM EGTA to the lysed cells. The K_d used for calibration was 224 nM. For desensitization experiments, cells were first stimulated with buffer or chemokine at different concentrations. As a second stimulus, chemokines were added at a concentration inducing a significant increase in the $[Ca^{2+}]_i$ after prestimulation with buffer. The percentage inhibition of the $[Ca^{2+}]_i$-increase in response to the second stimulus by prestimulation of the cells was calculated.

Inhibition of HIV-1 infection

The HIV-1 M-tropic strains BaL and SF162 were obtained through the MRC AIDS reagent project (Herts, UK). Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated by density gradient centrifugation (5,23) and stimulated with PHA at 1 µg/ml (Sigma, Bornem, Belgium) for 3 days at 37°C. The activated cells (PHA-stimulated blasts) were washed three times with PBS, and infected with a virus as described previously (Schols D. et al., 1997). HIV-1 infected or mock-infected PHA-stimulated blasts were cultured in the presence of 25 U/ml of IL-2 and varying concentrations of RANTES (1-68) or RANTES (3-68). Cell supernatant was collected at day 10 and HIV-1 core antigen in the supernatant was analysed by a p-24 Ag ELISA kit (DuPont/NEN Life Science Products, Brussels, Belgium).

Results

Identification and biological characterization of natural, NH_2-terminally truncated RANTES.

A different NH_2-terminally truncated form of human GCP-2 has been previously isolated (Proost P. et al., 1993). The least truncated GCP-2-form was cleaved beyond Pro at the penultimate position [GCP-2(3-75)]. Using a similar standard purification

procedure, the C-C chemokine RANTES was purified from peripheral blood leukocytes or sarcoma cells (Proost P. et al., 1996).

In particular, conditioned media from MG-63 or Malavu sarcoma cells induced with a cytokine mixture were fractionated to isolate natural chemokine variants. The chemokines were purified by subsequent antibody or heparin affinity chromatography, cation-exchange chromatography (mono S FPLC) and RP-HPLC, and immunoreactive forms were detected by specific chemokine ELISAs. On the cation-exchange column, IL-8 was found to elute in close proximity of RANTES (between 0.7 and 0.75 M NaCl). Nevertheless, both chemokines were separated from each other by RP-HPLC (RANTES and IL-8 eluting at 27.5% and 30% acetonitrile, respectively). Amino acid sequence analysis of the pure proteins confirmed that IL-8 occured in different NH_2-terminally truncated forms, which were previously isolated on the basis of their chemotactic activity (Van Damme J. et al., 1989). However, for RANTES only one single form was isolated, which was missing two NH_2-terminal residues compared to intact RANTES. In view of its predominant appearance, this RANTES(3-68) was analyzed in more detail to verify its chemotactic activity for monocytes and eosinophils. In particular, RANTES(3-68) was tested for chemotactic and/or intracellular Ca^{2+}-releasing activity and their biological potency was compared with that of the respective intact chemokines.

NH_2-terminal deletion of two residues from RANTES resulted in considerably decreased monocyte chemotactic and Ca^{2+}-releasing activities. Compared to intact natural RANTES (minimal effective dose of 3-10 ng/ml), natural RANTES(3-68) was totally inactive when tested at concentrations as high as 300 ng/ml in the Boyden microchamber (Figure 2). In addition, 10 times higher concentrations of natural RANTES(3-68), compared to RANTES(1-68), were necessary to obtain a similar Ca^{2+}-response (Figure 3).

CD26/DPP IV removes the NH_2-terminal dipeptides of chemokines

In order to investigate whether the aminopeptidase CD26/DPP IV could be responsible for the NH_2-terminal truncation of RANTES, the intact chemokine was incubated overnight with CD26/DPP IV, blotted to PVDF membranes, stained with Coomassie blue and subjected to automatic Edman degradation. CD26/DPP IV

treatment of RANTES resulted in the removal of the NH_2-terminal dipeptides. Parallel incubation of chemokine with buffer without CD26/DPP IV had no effect.

Since other chemokines contained the consensus sequence for CD26/DPP IV cleavage and since the NH_2-terminus of MCPs was shown to be crucial for biological activity (Gong J. et al., 1996 and Gong J. et al., 1995), MCP-1, MCP-2 and MCP-3 were also incubated with CD26/DPP IV.

After treatment, MCPs were blotted on PVDF membranes and Coomassie blue stained to confirm that a sufficient amount of protein was recovered for Edman degradation.

However, no NH_2-terminal sequence could be detected, indicating that CD26/DPP IV does not alter the NH_2-terminus of MCPs which is blocked for Edman degradation by a pyroglutamic acid.

Comparison of the biological activity of intact and CD26/DPP IV-treated RANTES.

Similar to natural RANTES(3-68), C-8 RP-HPLC purified, CD26/DPP IV-treated recombinant RANTES was inactive in Boyden microchamber chemotaxis experiments when used at concentrations up to 1 μg/ml, while a significant monocyte chemotactic response was detected with intact recombinant RANTES from 30 to 100 ng/ml onwards (Figure 2).

When the truncation effect was tested in the Ca^{2+}-mobilization assay, RANTES(3-68) induced a low but significant increase at 100 ng/ml. Intact RANTES, however, was already active at 10 ng/ml (Figure 3). In conclusion, although only two NH_2-terminal residues were removed, the monocyte chemotactic and Ca^{2+}-mobilizing potency of RANTES decreased 10 to 100-fold.

RANTES(3-68) is a natural chemotaxis antagonist for intact RANTES

In view of the inactivity of RANTES(3-68) in monocyte chemotaxis experiments, we tested whether this truncated RANTES might act as an antagonist. RANTES(3-68), at 1 μg/ml, almost completely (82 %) desensitized for the chemotactic effect of 100 ng/ml of intact RANTES (Table I).

When a 3-fold excess of RANTES(3-68) was added to the upper well, chemotaxis of THP-1 cells towards intact RANTES was inhibited by about 50-70 %. RANTES(3-68) at 300 ng/ml could still inhibit about 30 % of the chemotactic response towards an equal concentration of intact RANTES.

In Ca^{2+}-mobilization experiments with THP-1 cells (Figure 4), 30 ng/ml of intact RANTES could desensitize for the effect of 30 ng/ml of intact RANTES for 39 ±5 %. About ten-fold higher concentrations of RANTES(3-68) were necessary to obtain the same amount of desensitization. However, at 300 ng/ml, RANTES(3-68) by itself gave a significant Ca^{2+}-response. This Ca^{2+}-response was comparable to the response obtained with 30 ng/ml of intact RANTES.

TABLE I

RANTES(3-68) desensitizes monocyte chemotaxis induced by RANTES(1-68)[1]

Chemokine(ng/ml)		Chemotactic response (CI)					
Lower well	Upper well						% inhibition
RANTES (1-68)	RANTES (3-68)	A	B	C	D	mean ± SEM	mean ± SEM
300	1000	12.5	7.5	27.5	50.5	25 ± 10	67 ± 8
	300	22.0	20.5	72.5	79.5	49 ± 16	31 ±13
	0	41.0	46.0	71.5	97.0	64 ±1 3	0
100	1000	4.0	3.0	13.5	11.0	8 ± 3	82 ± 4
	300	7.5	7.0	29.0	33.0	19 ± 7	53 ± 11
	0	24.0	21.5	50.0	44.5	35 ± 7	0

[1] Results represent the chemotactic index (C.I.) of four (A to D) independent experiments (including mean ± SEM) and the percentage (%) inhibition (mean ± SEM of the % inhibition of the four experiments) of the chemotactic response towards RANTES(1-68) after preincubation of the THP-1 cells with inactive RANTES(3-68) or buffer.

Impaired chemotactic activity of RANTES(3-68) for human monocytes and eosinophils

In Table II the chemotactic potency of natural RANTES(3-68) is compared with that of the monocyte chemotactic protein MCP-3 and intact RANTES(1-68). It can be seen that MCP-3 and RANTES(1-68) are still chemotactic for freshly isolated peripheral blood monocytes at 3 ng/ml and 30 ng/ml, respectively, whereas natural RANTES(3-68) remained inactive at 100 ng/ml.

The reduced chemotactic potency of this natural variant, was confirmed with recombinant RANTES(3-68). Although weakly chemotactic for monocytes (at 1 µg/ml), purified recombinant RANTES(3-68) showed a specific activity which is 10-fold lower than that of intact recombinant RANTES.

Finally, the chemotactic potency of RANTES(3-68) was verified on human eosinophils, which were still responsive to 100 ng/ml of intact RANTES and 30 ng/ml of

MCP-3 (Figure 5). Similar to monocytes, eosinophil migration was only stimulated by RANTES(3-68) at 1 µg/ml.

TABLE II

Comparison of the monocyte chemotactic activity of RANTES(3-68) with RANTES(1-68) and MCP-3

conc. (ng/ml)	monocyte chemotactic activity [a] MCP-3	natRANTES (3-68)	recRANTES (1-68)	recRANTES (3-68)
1000	—[b]	—	3.6 ± 0.8 (6)	3.3 (1)
300	6.0 ± 1.2 (6)	—	—	—
100	—	1.1 ± 0.1 (3)	3.3 ± 0.4 (6)	1.0 (1)
30	6.9 ± 1.0 (6)	1.7 ± 0.2 (3)	—	—
10	—	1.9 ± 0.6 (3)	1.9 ± 0.4 (6)	< 1.0 (1)
3	4.1 ± 0.4 (6)	—	—	—

[a] mean chemotactic index (CI) ± SEM (n) on freshly isolated peripheral blood monocytes.
[b] not determined

RANTES(3-68) signals and desensitizes for RANTES(1-68) through CCR5, but not through CCR1 and CCR3

To explain the reduced chemotactic activity of RANTES(3-68), the capacity of this chemokine variant to bind and signal through the known receptors used by RANTES was verified.

HOS cells transfected with the chemokine receptors CCR1, CCR3 or CCR5 were used in a signaling assay measuring increases in the intracellular calcium concentration. At concentrations up to 300 ng/ml, RANTES(3-68) did not increase the $[Ca^{2+}]_i$ in HOS cells transfected with CCR1 (Table III) or CCR3 (data not shown), whereas 30 ng/ml and 100 ng/ml of intact RANTES was sufficient to induce an increase in $[Ca^{2+}]_i$ in CCR1 and CCR3 transfectants, respectively.

However, both intact and truncated RANTES were able to induce a significant rise in $[Ca^{2+}]_i$ in CCR5 transfectants at 30 ng/ml. Furthermore, by pre-incubation of

CCR5-transfected cells with a 3 to 10-fold excess of either RANTES(3-68) or intact RANTES, an equal inhibition (about 75%) of the $[Ca^{2+}]_i$ rise by a subsequent challenge with intact RANTES (100 ng/ml) was obtained (Figure 6).

In contrast, 300 ng/ml of RANTES(3-68) only marginally desensitized the calcium response of CCR1 and CCR3-transfected cells to 100 ng/ml of intact RANTES, whereas a 3-fold excess of intact RANTES as first stimulus almost completely inhibited the $[Ca^{2+}]_i$ rise in these cells by subsequent RANTES(1-68). It must be concluded that removal of two NH_2-terminal residues from RANTES has a significant impact on signal transduction in that the chemokine receptors CCR1 and CCR3 are no longer functionally recognized. Therefore, the impaired chemotactic potency of RANTES(3-68) can be explained by its inability to function through CCR1 and CCR3. In contrast, RANTES(3-68) fully retained the CCR5 signalling characteristic of intact RANTES. RANTES(3-68) can be anti-inflammatory by competing with intact RANTES, but may still function as an HIV-inhibitor by retaining its capacity to bind CCR5.

TABLE III

Calcium mobilization by RANTES forms in CCR1 and CCR5 transfectants

chemokine	conc. (ng/ml)	increase in $[Ca^{2+}]_i$ (nM)[a]	
		CCR1	CCR5
RANTES(1-68)	300	133 ± 5 (3)	96 ± 1 (2)
	100	100 ± 28 (3)	60 ± 4 (2)
	30	25 ± 8 (3)	24 ± 2 (2)
	10	<15 (2)	<15 (1)
RANTES(3-68)	300	19 ± 9 (3)	119 ± 5 (2)
	100	<15 ± 0 (3)	76 ± 4 (2)
	30	<15 (2)	56 ± 13 (2)
	10		<15 (1)

[a] the mean increase in $[Ca^{2+}]_i$ in nM ± SEM of two or more independent experiments is shown.

Inhibition of CC chemokine-induced chemotaxis by RANTES(3-68) in human monocytic cells

To verify whether inhibition of CC chemokine signaling by RANTES(3-68) also occurred in monocytic cells, inhibition experiments were conducted in THP-1 cells. It was evidenced that RANTES(3-68) showed a 10-fold reduction in potency to increase the $[Ca^{2+}]_i$ in monocytic cells compared to intact RANTES (data not shown). In addition, the chemotactic effect of intact RANTES (30 ng/ml) on monocytic cells was inhibited (71%) by incubating the test cells with 300 ng/ml RANTES(3-68) as shown in Table IV.

Furthermore, RANTES(3-68) reduced the chemotactic response to other CC chemokines, including monocyte chemotactic protein-3 (MCP-3) (67%), macrophage inflammatory protein-1a (MIP-1α) (61%) and MIP-1β (80%).

This illustrates that RANTES(3-68) functions as a broad spectrum inhibitor of monocytic cell migration induced by other CC chemokines.

TABLE IV

Inhibition of monocytic cell chemotaxis towards CC chemokines by RANTES(3-68)

		inhibition of THP-1 cell chemotaxis		
chemokine[a)]	conc. (ng/ml)	buffer[b,c)]	RANTES(3-68)[b,c)]	% inhibition[d)]
RANTES	30	19.0 ± 6.6	3.7 ± 0.6	71 ± 16
MCP-3	30	48.5 ± 9.3	24.9 ± 2.0	45 ± 10
MCP-3	3	7.6 ± 2.5	3.1 ± 0.8	67 ± 13
MIP-1α	30	6.2 ± 2.4	3.0 ± 1.1	61 ± 22
MIP-1β	300	4.3 ± 1.0	1.9 ± 0.6	80 ± 12
control		1.5 ± 0.5	1.0 ± 0.5	

a) RANTES, MCP-3, MIP-1α, MIP-1β and buffer were added as chemoattractants to the lower wells of the microchamber.

b) the upper wells of the microchamber were filled with THP-1 cells preincubated (10 min, 37°C) with 300 ng/ml RANTES(3-68) or with buffer.

c) mean CI ± SEM of four independent experiments.

d) inhibition of migration induced by intact chemokines in the presence of RANTES(3-68) at 300 ng/ml.

CD26-specific truncation of RANTES is necessary for its antiviral activity.

The effects of the different forms of RANTES were first evaluated against two different M-tropic HIV-1 strains (BaL and SF162) in human PBMC derived from healthy blood donors. The IC_{50} of the intact RANTES(1-68) against the BaL strain was 3.4 nM and for RANTES(3-68) the IC_{50} was 0.39 nM. The IC_{90} value for RANTES(1-68) was 71 nM, which was about 10-fold the IC_{90} for RANTES(3-68). Against the SF162 strain when evaluated in PBMC, RANTES(1-68) (IC_{50}: 23 nM; IC_{90} : 95 nM) was more than 10-fold less active than RANTES(3-68) (IC_{50}: 2 nM; IC_{90} : 8.2 nM) (Table V). The concentration-dependent effects of both chemokines at concentrations ranging from 133 down to 0.2 nM against HIV-1 SF162 replication in PBMC are shown in Figure 8. A concentration of 5.2 nM RANTES(3-68) was clearly effective in reducing virus replication, whereas RANTES(1-68) was inactive at this concentration. No difference in antiviral activity was noticed between intact RANTES obtained from PeproTech or R&D Systems.

The striking difference in antiviral activity between the two forms of RANTES became even more apparent when tested in the human CCR5 transfected cells. In U78.CD4.CCR5 cells, the IC_{50} for RANTES(1-68) and RANTES(3-68) against the BaL strain was 21 nM and 0.65 nM, respectively. The IC_{90} for RANTES(1-68) was more than 133 nM, whereas the IC_{90} for RANTES(3-68) was 63 nM. Also in Table V, it is shown that RANTES(1-68) was virtually inactive in the HOS.CD4.CCR5 cells (IC_{50} > 133 nM), whereas RANTES(3-68) is a potent inhibitor of HIV-1 BaL replication in these cells (IC_{50}: 5.5 nM). However, no IC_{90} values were reached for both forms of RANTES in these cells.

The antiviral activity of RANTES is dependent on the presence of membrane bound or soluble CD26 (sCD26).

The CD26 expression on the two different CCR5-transfected cell lines and on freshly isolated PBMC was evaluated. HOS transfectants were negative for CD26 expression as determined by flow cytometric analysis, whereas U87 transfectants stained weakly but significantly positive with the anti-CD26 mAb (Figure 9). In addition, a

subpopulation of freshly isolated PMBC was found to be strongly positive for CD26 expression (Figure 9).

The concentration-dependent effect of RANTES(1-68) and RANTES(3-68) on viral p24 Ag production by the BaL strain in HOS.CD4.CCR5 transfected cells in presence of sCD26 is shown in Figure 10. Addition of sCD26, together with RANTES(1-68) at the start of the HIV infection, significantly enhanced the antiviral activity of the intact RANTES in HOS.CD4.CCR5 cells. When sCD26 at 50 U/l was added together with RANTES, an IC_{50} of 13 nM of RANTES was obtained. The addition of sCD26 alone had no effect on virus replication. The addition of sCD26 to RANTES(3-68) did also not change the antiviral activity of RANTES(3-68) (data not shown). Thus, the presence of CD26 is essential for intact RANTES to become antivirally active.

Amino terminal truncation of natural MIP-1α does not affect its anti-HIV-1 chemotactic and Ca^{2+} mobilising activity.

Since the majority of natural MIP-1α is NH_2 terminally truncated (four amino acids), we investigated whether this truncated MIP-1α(5-70) had an altered HIV-1 inhibitory capacity. In contrast with the results obtained for RANTES, no significant differences were detected for the IC_{50} values of intact MIP-1α and MIP-1α(5-70) in PMBC or CCR5-transfected cells (Table V, Figure 8). In addition, intact MIP-1α and truncated MIP-1α(5-70) were compared in chemotaxis and intracellular Ca^{2+}-mobilization assays on THP-1 monocytic cells. Table VI demonstrates that the minimal effective dose of MIP-1α(5-70) inducing a rise in the $[Ca^{2+}]_i$ was only slightly lower than intact MIP-1α. Furthermore, although maximal migration obtained with 0.13 nM in the chemotaxis assay was higher for intact MIP-1α, the minimal affective concentrations of both MIP-1α isoforms were rather similar. Taken together, it must be concluded that NH_2 terminal processing of MIP-1α in contrast to RANTES, only minimally weakens its inflammatory and anti-HIV-1 activity.

TABLE V. Anti-HIV-1 activity of RANTES and MIP-1α in PHA-stimulated PMBC, U87.CD4.CCR5 cells.

	RANTES(1-68)		RANTES(3-68)		MIP-1α(1-70)		MIP-1α(5-70)	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
PMBC								
BaL	3.4	71	039	6.9	1.9	62	1.6	13
SF162	23	95	2.0	8.2	3.1	30	3.6	32
U87.CD4.CCR5								
BaL	21	>130	0.65	63	ND	ND	ND	ND
HOS.CD4.CCR5								
BaL	>130	>130	5.5	>130	32	>130	21	>130

Virus yield was monitored in the cell-free supernatant 8-12 days after infection by viral p24 Ag ELISA. The mean IC_{50}s and IC_{90}s (in nM) are shown. The data represent the means of two to four independent experiments. The value marked by ">130" indicates that 50% or 90% inhibition is not achieved at 130 nM. ND, not done.

TABLE VI Lack of difference in biological potency between intact and truncated MIP-1α.

	Chemotaxis*		Increase in $[Ca^{2+}]_i$†	
Chemokine	nM	C.I	nM	nM $[Ca^{2+}]_i$
MIP-1α(1-70)	1.3	8.5 ± 3.3	3.9	240/195
	0.13	22.2 ± 4.4	0.39	120/130
	0.013	5.7 ± 1.6	0.34	30/14
	0.0013	4.0 ± 3.1		
MIP-1α(5-70	1.3	14.2 ± 0.4	4.0	196/178
	0.13	11.4 ± 2.9	0.4	71/33
	0.013	3.8 ± 1.4	0.04	10/<10
	0.0013	2.1 ± 0.6		

* Migration of monocyte THP-1 cells through 5.0 μm pores in the microchamber. Resuls are mean ± of three independent experiments.

† Detection of the $[Ca^{2+}]_i$ increase in THP-1 cells. Results of two independent experiments are shown.

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CLAIMS

1. Amino-terminally truncated RANTES, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3 or 1-4 of the naturally-occurring RANTES and having chemokine antagonistic activity.

2. Amino-terminally truncated RANTES according to claim 1, lacking NH_2-terminal amino acids corresponding to amino acid residues 1-2 of the naturally-occurring RANTES and having a chemokine antagonistic activity

3. Amino-terminally truncated RANTES according to claim 1, having the amino acid sequence of SEQ ID NO: 2.

4. Amino-terminally truncated RANTES, according to one or more of the preceding claims, in a glycosylated form.

5. DNA molecules comprising the DNA sequences coding for the amino-terminally truncated RANTES of the invention according to one or more of the preceding claims, including nucleotide sequences substantially the same.

6. An expression vector which comprises the DNA molecule of any claim 5.

7. A host cell comprising the expression vector of claim 6.

8. A recombinant process for preparing any of the proteins from claim 1 to 4, comprising culturing in an appropriate culture medium the cells of claim 7.

9. A protein according to any of the claims from 1 to 4 for use as medicament.

10.Use of a protein according to any of the claims from 1 to 4, in the manufacture of a medicament for the therapy and/or diagnosis of diseases, in which an antagonistic activity of the chemokine effects is required.

11.Use according to claim 10, in the manufacture of a medicament for the treatment of inflammatory diseases, HIV-infection, angiogenisis- and hematopoiesis-related diseases, and tumors.

12.A pharmaceutical composition comprising the protein according to any of the claims from 1 to 4 together with one or more pharmaceutically acceptable carriers and/or excipients.

13.Use of CD26/DPP IV in the therapy and/or diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required.

14.Use according to claim 13, for the treatment of inflammatory, immune and infectious diseases.

ABSTRACT

The present invention relates to amino-terminally truncated RANTES, lacking NH_2-terminal amino acids corresponding to amino acid residues 1, 1-2, 1-3 or 1-4 of the naturally-occurring RANTES and having chemokine antagonistic activity, as well as cDNA sequences encoding them, their use in therapy and/or in diagnosis of the diseases, in which an antagonistic activity of the chemokine effects is required, and pharmaceutical compositions comprising them.

RANTES

-23 1 ↓

MKVSAARLAV ILIATALCAP ASASPYSSDT TPCCFAYIAR PLPRAHIKEY FYTSGKCSNP AVVFVTRKNR QVCANPEKKW VREYINSLEM S

68

Figure 1

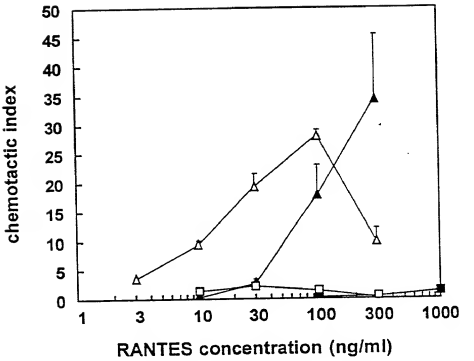

chemotactic index
50
45
40
35
30
25
20
15
10
5
0
1
3
10
30
100
300
1000
RANTES concentration (ng/ml)

Figure 2

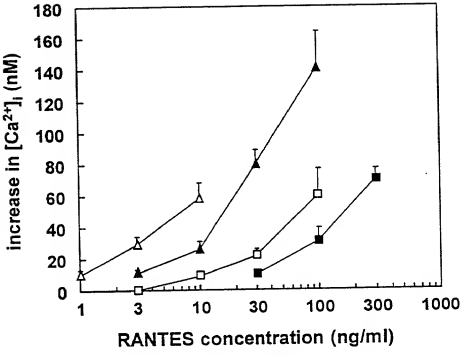

180
160
140
120
100
80
60
40
20
0
increase in [Ca2+]i (nM)
1
3
10
30
100
300
1000
RANTES concentration (ng/ml)

Figure 3

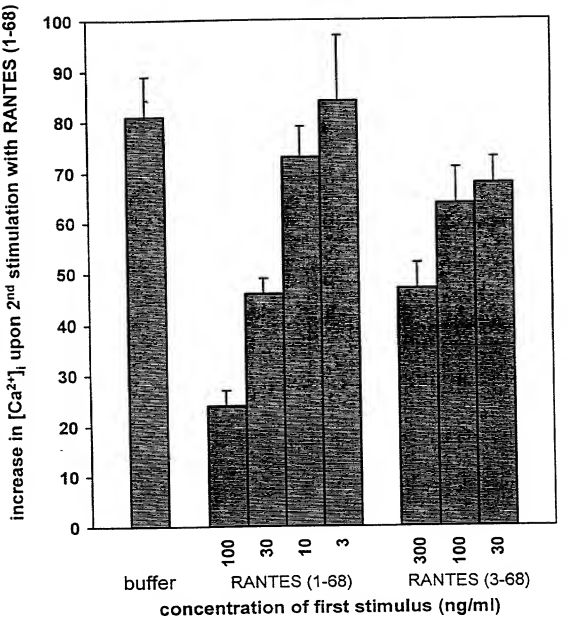
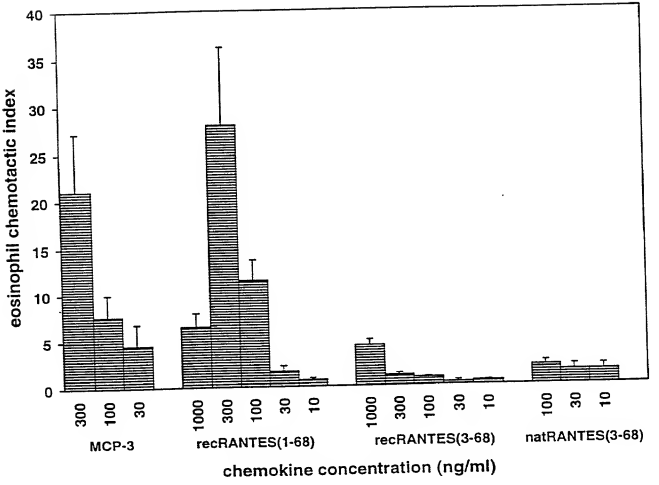

increase in $[Ca^{2+}]_i$ upon 2nd stimulation with RANTES (1-68)
100
90
80
70
60
50
40
30
20
10
0
100
30
10
3
300
100
30
buffer
RANTES (1-68)
RANTES (3-68)
concentration of first stimulus (ng/ml)

Figure 4


eosinophil chemotactic index
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35
30
25
20
15
10
5
0
300
100
30
1000
300
100
30
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1000
300
100
30
10
100
30
10
MCP-3
recRANTES(1-68)
recRANTES(3-68)
natRANTES(3-68)
chemokine concentration (ng/ml)

Figure 5

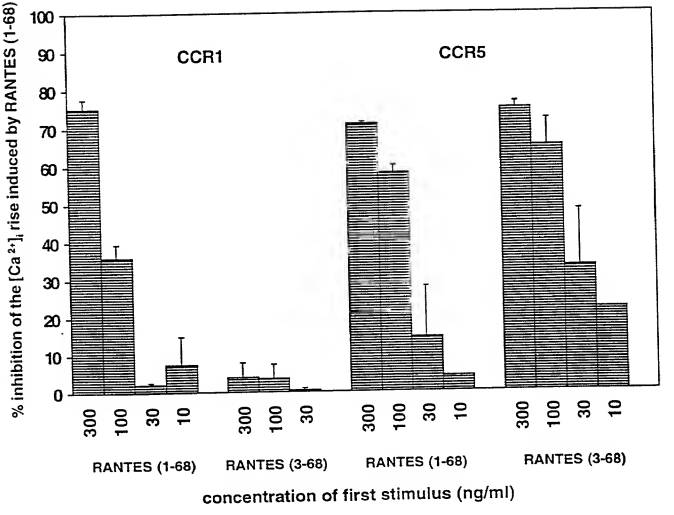
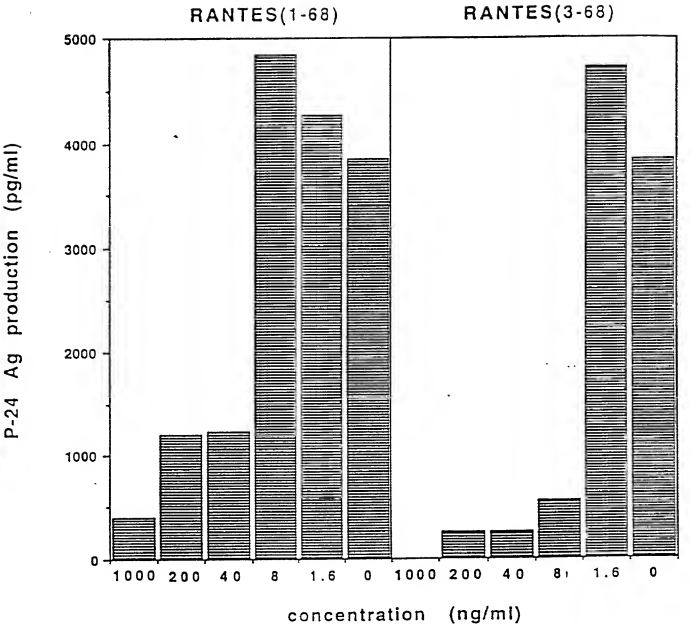

% inhibition of the [Ca 2+]i rise induced by RANTES (1-68)
100
90
80
70
60
50
40
30
20
10
0
CCR1
CCR5
300
100
30
10
300
100
30
300
100
30
10
300
100
30
10
RANTES (1-68)
RANTES (3-68)
RANTES (1-68)
RANTES (3-68)
concentration of first stimulus (ng/ml)

Figure 6


RANTES(1-68)
RANTES(3-68)
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4000
3000
2000
1000
0
P-24 Ag production (pg/ml)
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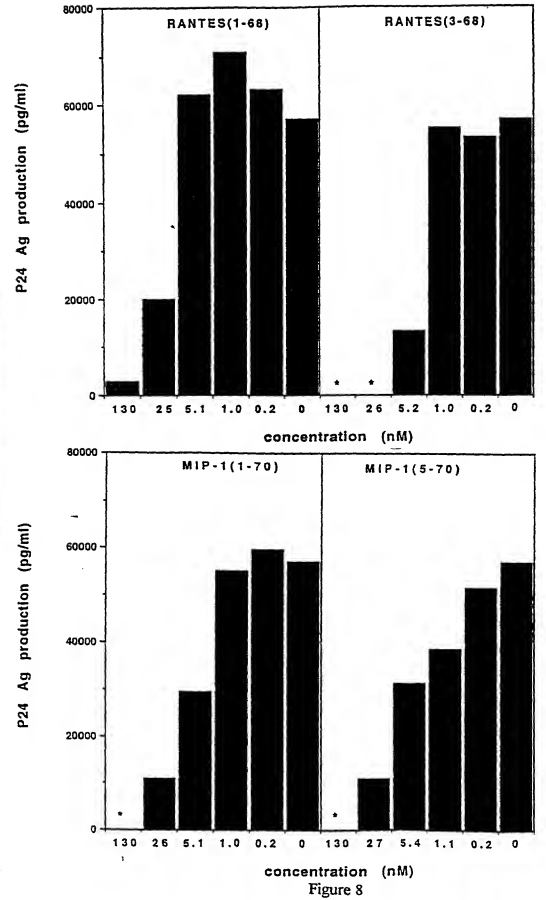
concentration (ng/ml)

Figure 7


RANTES(1-68)
RANTES(3-68)
P24 Ag production (pg/ml)
80000
60000
40000
20000
0
130 25 5.1 1.0 0.2 0
130 26 5.2 1.0 0.2 0
concentration (nM)
MIP-1(1-70)
MIP-1(5-70)
P24 Ag production (pg/ml)
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60000
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0
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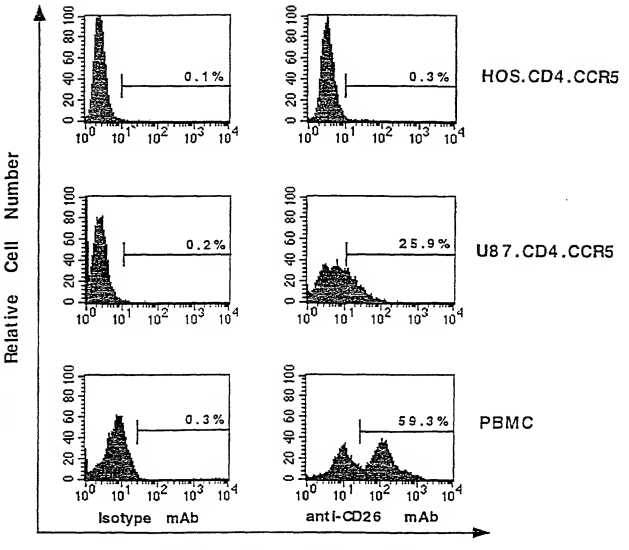
concentration (nM)

Figure 8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0.3%
HOS.CD4.CCR5
0.2%
25.9%
U87.CD4.CCR5
0.3%
59.3%
PBMC
Isotype mAb
anti-CD26 mAb
Relative Red Fluorescence

Figure 9

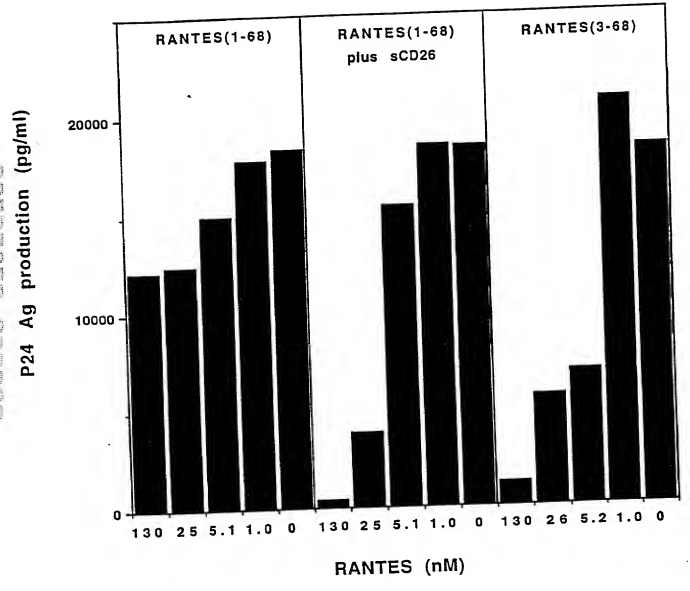
RANTES(1-68)
RANTES(1-68)
plus sCD26
RANTES(3-68)
P24 Ag production (pg/ml)
20000
10000
0
130 25 5.1 1.0 0
130 25 5.1 1.0 0
130 26 5.2 1.0 0
RANTES (nM)

Figure 10

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: APPLIED RESEARCH SYSTEMS ARS HOLDING N.V.
(B) STREET: 14 JOHN B. GORSIRAWEG
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(E) COUNTRY: THE NETHERLANDS ANTILLES
(F) POSTAL CODE (ZIP): NONE
(G) TELEPHONE: 599-9-639300
(H) TELEFAX: 599-9-614129

(ii) TITLE OF INVENTION: AMINO-TERMINALLY TRUNCATED RANTES AS CHEMOKINE ANTAGONISTS

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 91 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION:1..68

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

```
Met Lys Val Ser Ala Ala Arg Leu Ala Val Ile Leu Ile Ala Thr Ala
            -20                 -15                 -10

Leu Cys Ala Pro Ala Ser Ala Ser Pro Tyr Ser Ser Asp Thr Thr Pro
        -5                  1               5

Cys Cys Phe Ala Tyr Ile Ala Arg Pro Leu Pro Arg Ala His Ile Lys
10                  15                  20                  25

Glu Tyr Phe Tyr Thr Ser Gly Lys Cys Ser Asn Pro Ala Val Val Phe
                30                  35                  40
```

Val Thr Arg Lys Asn Arg Gln Val Cys Ala Asn Pro Glu Lys Lys Trp
45 50 55

Val Arg Glu Tyr Ile Asn Ser Leu Glu Met Ser
60 65

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Tyr Ser Ser Asp Thr Thr Pro Cys Cys Phe Ala Tyr Ile Ala Arg Pro
1 5 10 15

Leu Pro Arg Ala His Ile Lys Glu Tyr Phe Tyr Thr Ser Gly Lys Cys
20 25 30

Ser Asn Pro Ala Val Val Phe Val Thr Arg Lys Asn Arg Gln Val Cys
35 40 45

Ala Asn Pro Glu Lys Lys Trp Val Arg Glu Tyr Ile Asn Ser Leu Glu
50 55 60

Met Ser
65